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# Phycourobilin in Trichromatic Phycocyanin from Oceanic Cyanobacteria Is Formed Post-translationally by a Phycoerythrobilin Lyase-Isomerase*[S]

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**Most cyanobacteria harvest light with large antenna complexes called phycobilisomes. The diversity of their constituting phycobiliproteins contributes to optimize the photosynthetic capacity of these microorganisms. Phycobiliprotein biosynthesis, which involves several post-translational modifications including covalent attachment of the linear tetrapyrrole chromophores (phycobilins) to apoproteins, begins to be well understood. However, the biosynthetic pathway to the blue-green-absorbing phycourobilin ($\lambda_{max} \sim 495$ nm) remained unknown, although it is the major phycobilin of cyanobacteria living in oceanic areas where blue light penetrates deeply into the water column. We describe a unique trichromatic phycocyanin, R-PC V, extracted from phycobilisomes of *Synechococcus* sp. strain WH8102. It is evolutionarily remarkable as the only chromoprotein known so far that absorbs the whole wavelength range between 450 and 650 nm. R-PC V carries a phycourobilin chromophore on its α-subunit, and this can be considered an extreme case of adaptation to blue-green light. We also discovered the enzyme, RpcG, responsible for its biosynthesis. This monomeric enzyme catalyzes binding of the green-absorbing phycoerythrobilin at cysteine 84 with concomitant isomerization to phycourobilin. This reaction is analogous to formation of the orange-absorbing phycoviolobilin from the red-absorbing phycocyanobilin that is catalyzed by the lyase-isomerase PecE/F in some freshwater cyanobacteria. The fusion protein, RpcG, and the heterodimeric PecE/F are mutually interchangeable in a heterologous expression system in *Escherichia coli*. The novel R-PC V likely optimizes rod-core energy transfer in phycobilisomes and thereby adaptation of a major phytoplankton group to the blue-green light prevailing in oceanic waters.**

To perform photosynthesis, the main energetic basis for life on earth, phototrophic organisms have to cope with large spatial and temporal variations of light conditions. A major evolutionary step in meeting this challenge was the development of light-harvesting complexes, the most variable part of the photosynthetic apparatus (1). By binding a large number of chromophores, these antennas can considerably enhance the photon absorption capacity of reaction centers that are responsible for the conversion of solar energy into chemical energy. Pigmented proteins associated with light-harvesting complexes also fill (at least partially) the large gap between the absorption bands of reaction center chlorophylls (*e.g.* ~440 and 680 nm for chlorophyll *a* found in most oxygenic organisms). Antennas also transport the excitons with minimal loss and transduce high energy excitons into the low energy ones required by the reaction centers (1, 2). They do not only vary among the different organisms but also with time within individual organisms, thereby providing the flexibility needed by the photosynthetic apparatus to work efficiently under varying ambient conditions.

Cyanobacteria, which contribute a substantial fraction of global photosynthesis (3), evolved a particularly sophisticated and dynamic antenna complex, the phycobilisome (PBS)[4] (4, 5). This extramembranous structure with a size of several MDa is mainly composed of the deeply colored and intensely fluo-

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[4] The abbreviations used are: PBS, phycobilisome; PC, phycocyanin; C-PC, C-type phycocyanin; PCB, phycocyanobilin; R-PC, R-phycocyanin (prefix R originally referred to as "rhodophytes," but now designates spectral type); RpcA, apo-α-subunit of R-PC; RpcG, fused PUB lyase (EF-type); IEF, isoelectric focusing; PE, phycoerythrin; PEB, phycoerythrobilin; PEC, phycoerythrocyanin; PecA, apo-α-PEC; PecE, PecF, subunits of PVB:α-PEC lyase-isomerase; PUB, phycourobilin; PVB, phycoviolobilin; HPLC, high pressure liquid chromatography.

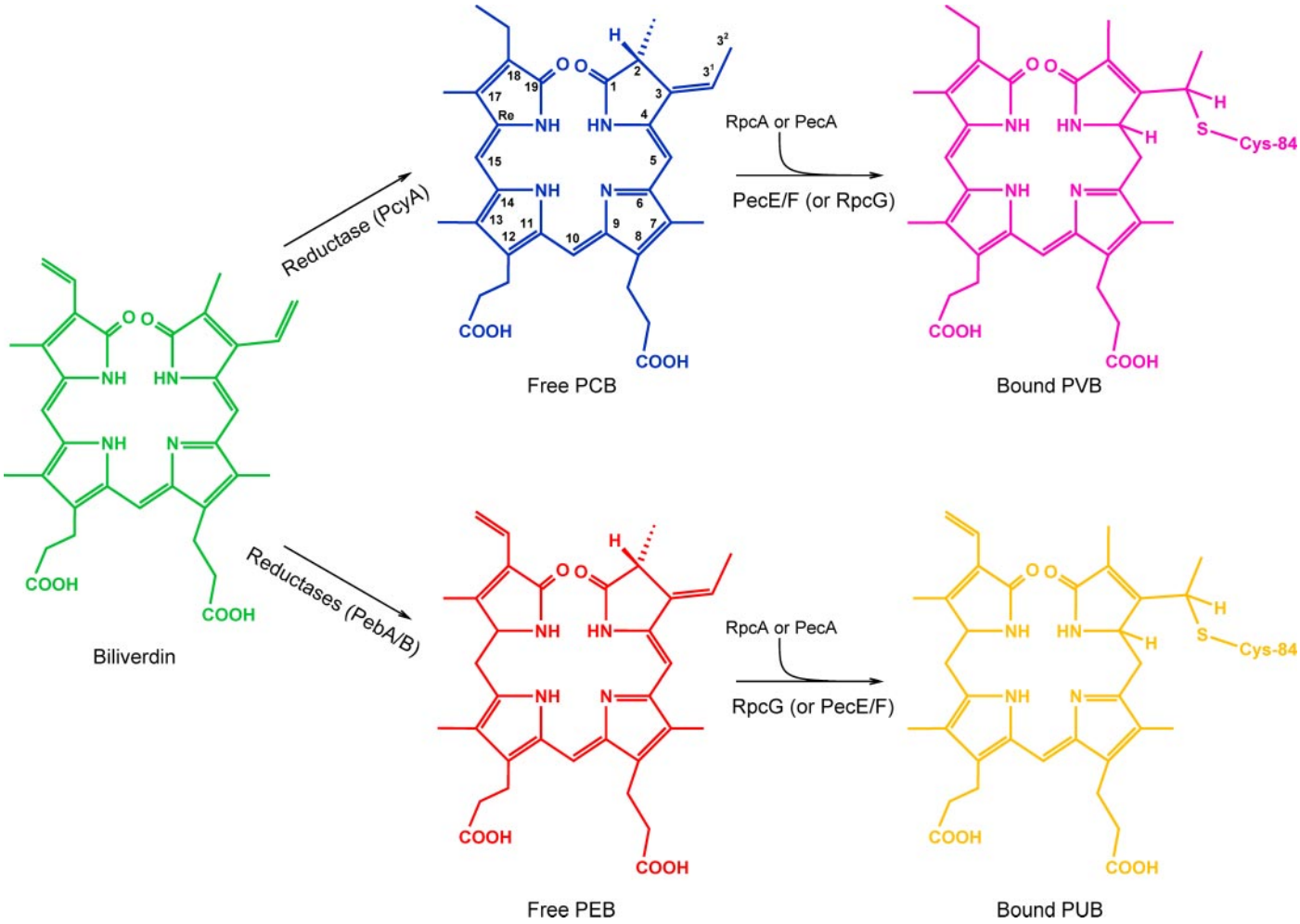


FIGURE 1. **Chromophore structures and biosynthesis of biliprotein chromophores.** The biosynthesis of the yellow-absorbing PVB and the blue-green-absorbing PUB from the orange-absorbing PCB and the green-absorbing PEB, respectively, during post-translational attachment of chromophores is illustrated. The colors and geometries indicate those of the uncoupled chromophores. They can vary considerably in the native chromoproteins due to non-covalent interactions.

rescing phycobiliproteins. A single PBS, generally composed of a central core and 6–8 radiating rods, contains hundreds of linear tetrapyrrole chromophores (phycobilins) that are covalently attached to their apoproteins. The remarkably wide diversity of PBSs found in nature is due to the large number of possible combinations of phycobiliproteins with various pigmentations that constitute the rods. Marine *Synechococcus*, the second most abundant oxyphototrophic organism on Earth after *Prochlorococcus* (3, 6), is the cyanobacterial group in which the largest diversity of PBS rod composition (and hence pigmentation) can be found (7). This is particularly useful to enable this group to cope with the variety of light environments encountered in marine ecosystems.

PBSs can, at least in part, be reversibly dissociated (8–10). Progress in understanding PBS assembly was slow, however, due to the complex and poorly understood post-translational modifications of the phycobiliproteins required prior to assembly. Only after maturation can phycobiliproteins form spontaneously trimers, which are then integrated into the PBSs by interaction with specific structural proteins, so-called linker polypeptides. These post-translational modifications reactions include the covalent attachment of 1–4 chromophores to each individual apoprotein (11–20), methylation of an asparagine residue (21–23), and cleavage of N-terminal methionine residue (5, 24) (see UniProtKB/Swiss-Prot entry Q1XDQ2).

Of the three major phycobilins, the red-absorbing phycocyanobilin (PCB) and the green-absorbing phycoerythrobilin (PEB) are generated by specialized reductases from biliverdin (Fig. 1), the cleavage product of the heme macrocycle (26). Free PCB and PEB molecules are then attached by phycobilin lyases to specific binding sites on the phycobiliprotein subunits (18). Biosynthesis of a third chromophore, phycourobilin (PUB), which has never been found in free form, was until now enigmatic. Phycobiliproteins from all marine cyanobacteria adapted to oceanic waters, including *Synechococcus*, *Crocosphaera*, and *Trichodesmium*, are particularly rich in PUB, probably making it the most abundant phycobilin in the ocean (7, 27–30). Indeed, this chromophore absorbs efficiently blue-green light ($\lambda_{max}$ ~495 nm), a wavelength range prevailing in open oceanic subsurface waters and which is only poorly absorbed by chlorophyll *a*. Thus, elucidating the PUB biosynthesis process has been a challenge for many years.

So far, only three types of phycocyanin were known in marine *Synechococcus* spp., and none of them contained PUB. Among

the 11 recently sequenced strains (31), two (WH5701 and RS9917) have PBSs with rods entirely constituted of C-type phycocyanin (C-PC (7)), a form frequently found in freshwater cyanobacteria (5). This C-PC binds PCB at all three available chromophore binding sites: α-84, β-82, and β-153. Several phycoerythrin-containing marine *Synechococcus* strains possess phycocyanin of the R-type that carry PEB either at β-153 only (R-PC III), as in WH7805, or both at α-84 and at β-153 (R-PC II), as in WH7803 (5, 32, 33). Using comparative genomics, candidate genes were recently retrieved that code for the different phycobilin lyases required for catalyzing the chromophorylation of these various phycocyanins (7). For the C-PC, it was demonstrated in freshwater cyanobacteria (15–20) that three phycobilin lyases, the heterodimeric CpcE/F, the monomeric CpcS (also sometimes found in heterodimer with CpcU), and the monomeric CpcT, are needed to catalyze the attachment of PCB at α-84, β-84, and β-155, respectively (note that the positions of the chromophore binding sites slightly differ between the α-phycocyanin of freshwater and marine cyanobacteria), and all three sequences have clear orthologs in the WH5701 and RS9917 genomes. WH7805 contains phycobilin lyase genes (called *rpcE-F*) that are homologous to *cpcE-F*, and it has been suggested that they could encode a phycocyanin α-84 PEB lyase (34). In WH7803, the *cpcT* gene is missing and replaced by *rpcT*, which has been proposed to encode a phycocyanin β-153 PEB lyase (7). In four sequenced marine *Synechococcus*, including two oceanic strains with a particularly high PUB content, WH8102 and CC9605, and two type IV chromatic adapters, RS9916 and BL107, the *rpcE-F* operon is replaced by a single gene, *rpcG* (7). This gene appears to encode a fusion protein, and its N and C termini show much higher homology to *Nostoc (Anabaena) sp.* PCC 7120 PecE and PecF, respectively, which form the heterodimeric phycoerythrocyanin α-84 PCB lyase-isomerase, than to CpcE/F or RpcE/F lyases from other marine *Synechococcus*. Moreover, the RpcG C terminus carries a short motif (NHCQGN) that has been assigned an isomerase function in the F-subunit of PecF (35). This motif is responsible for the concomitant isomerization of the A-ring of PCB to generate the PVB chromophore. The analogous isomerization starting from free PEB would thus be expected to generate a cysteine-bound PUB chromophore (Fig. 1). This hypothesis led us to explore the function and specificity of the putative lyase-isomerase RpcG from two marine *Synechococcus* strains by a heterologous approach in *Escherichia coli* and to investigate the implications for phycobiliprotein pigmentation.

## EXPERIMENTAL PROCEDURES

*Preparation of Intact Phycobilisomes*—Marine *Synechococcus* spp. strains WH8102 and RS9916 were grown in PCR-S11 medium under continuous white light as described previously (36). Extraction of intact PBSs was carried out with 0.75 M phosphate buffer on a sucrose density gradient as described previously (37). PBSs used for purifying R-PC V were concentrated by ultracentrifugation, and pellets were either used immediately or kept frozen at −30 °C until use.

*Isolation of the Trichromatic Phycocyanin, R-PC V*—PBS pellets were solubilized in Hepes buffer (10 mM, pH 7.2) containing NaCl (5 mM), Pefabloc (1 mM), EDTA (1 mM), and β-mercaptoethanol (1%, v/v). PBS dissociation was allowed for 90 min at 0 °C. Individual phycobiliproteins were separated by non-denaturing isoelectric focusing (IEF) using 6% polyacrylamide (30% acrylamide, 1.6% bisacrylamide) tube gels, 4 mm in diameter, containing a convenient mixture of ampholytes (4% Servalyt 4–6, 1% Servalyt 3–7). Protein (200 μg) was loaded on the gel, and the isoelectric focusing was achieved with NaOH (20 mM) as a cathode buffer and phosphoric acid (0.045%) as the anode buffer (36). Voltage was gradually increased from 150 to 400 V by 50-V steps every 45 min. Phycocyanin was found focusing on the basic part of the gel, as a violet purple band, near various PE complexes that were all more acidic. The allophycocyanin components, focusing at lower acidic pH, were lost under these conditions. Bands of interest were cut out and kept frozen until further characterization.

*Subunit Isolation by Denaturing Isoelectric Focusing and Mass Spectrometry*—Denaturing polyacrylamide gels were prepared in 4-mm tubes as described above, the only modification being the addition of urea (8 M). Bands containing native biliproteins obtained by non-denaturing IEF were cut into small pieces and incubated *in situ*, on the top of urea acrylamide gels, in urea (9 M) containing mercaptoethanol (1% v/v) for 45 min, before starting the IEF in the same conditions as described above. All operations were made under dim light or, when possible, in complete darkness. Colored bands were cut out at the end of electrophoresis and frozen until use.

For mass spectrometric characterization of polypeptides, bands were cut out from IEF gels and fragmented into small pieces, denatured with 6% lithium dodecyl sulfate denaturation buffer, and loaded on lithium dodecyl sulfate-PAGE plate gels as described (37). The Coomassie Blue G 250-stained bands of phycobiliprotein subunits were analyzed by mass spectrometry using the facility at the "Unité de Phytopharmacie et Médiateurs Chimiques," Institut Nationale de la Recherche Agronomique (INRA) Versailles, France. Details on mass spectrometry experimental procedures can be found elsewhere (36).

*In-gel Spectroscopic Analyses of Separated Biliproteins*—Absorption spectra were obtained with a model DW2 spectrophotometer (Aminco Chance, Bogart, GA) using fragments of acrylamide gels containing the separated biliproteins. Polyacrylamide ampholyte gel fragments, with or without 8 M urea, were used as a blank. For recording the absorption spectra of denatured α- and β-subunits of R-PC V, samples were soaked for 15 min in acidic urea (8 M, acidified with HCl to pH 3) before recording the spectra.

*Gene Cloning*—Cloning and expression followed generally the standard procedures (38). The genes *cpcB* and *pecB* were PCR-amplified from *Fischerella sp.* PCC7603 (*Mastigocladus laminosus*), subsequently producing *cpcB*(C84S), *cpcB*(C155I), *pecB*(C84A), and *pecB*(C155I) via site-directed mutation (39); *cpcA*, *cpcE*, *cpcF*, *cpcS*, *cpcT*, *ho1*, *pcyA*, *pecA*, *pecE*, *pecF* from *Nostoc sp.* PCC 7120 (19, 40); and *cpeA1*, *cpeB*, *pebA*, and *pebB* from *Tolypothrix* (*Calothrix*) sp. PCC 7601 and then subsequently producing *cpeA*(C139S) and *cpeB*(C48A/C59S/C165S) via site-directed mutation (20). The genes *cpeA2*, *rpcG*, *mpeA*, and *rpcA* from *Synechococcus* sp. WH8102 and the genes *pebA*, *pebB*, *ho1*, *rpcA*, and *rpcG* from *Synechococcus* sp. RS9116 were PCR-amplified with the respective primers (supplemental

Table S1), then cloned into pBluescript SK (Stratagene, Beijing, China) or TOPO-TA cloning vector (Invitrogen), and then subcloned into pET-30 (Novagen, Munich, Germany). For application of the multiplasmid expression-chromophorylation system in *E. coli* (20), the above cloned genes were constructed into the corresponding duet plasmids (supplemental Table S2).

*Heterologous Gene Expression*—Duet plasmids were transformed together into BL21(DE3) cells under the appropriate antibiotic selections (chloramphenicol for pACYC-derivative, streptomycin for pCDF-derivative, kanamycin for pCOLA-derivative or pET30-derivative, ampicillin for pETDuet-derivative, see supplemental Table S2). To test the chromophorylation of the various apoproteins (CpcA, CpcB(C84S), CpcB(C155I), PecA, PecB(C84A), PecB(C155I), CpeA1, CpeA(C139S), CpeB, CpeB(C48A/C59S/C165S), and CpeA2, MpeA, RpcA), the respective encoding plasmid was transformed together with PCB- or PEB-producing plasmids (pACYC-*ho1-pcyA* for PCB, pCDF-*ho1-pebB* plus pACYC-*pebA* for PEB) and one or two of the lyase-producing plasmids into *E. coli* BL21(DE3) (supplemental S2). In the control experiments, plasmids containing lyase genes were omitted from the transformations. For chromophorylation in *E. coli*, cells were grown at 16–20 °C, depending on the genes to be expressed. 18–24 h after induction with isopropyl-1-thio-β-D-galactopyranoside (1 mM), cells were collected by centrifugation, washed twice with doubly distilled water, and stored at −20 °C until use.

*Isolation of Heterologously Synthesized Chromoproteins*—For isolation and purification of chromophorylated proteins, *E. coli* cells were suspended is Tris-HCl buffer (20 mM, pH 8) containing NaCl (300 mM) and imidazole (5 mM) and lysed by ultrasonication. The lysate was then centrifuged twice (10 min, 10,000 × *g*) to remove cell debris, and tagged proteins were isolated from the supernatant by $Ni^{2+}$ chromatography as described previously (19, 41). If necessary, the affinity-enriched proteins were further purified by fast protein liquid chromatography (Amersham Biosciences, Shanghai, China) over a DEAE FF column developed with a gradient of 0–0.6 M NaCl in potassium phosphate buffer (20 mM, pH 7.0).

*Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*—SDS-PAGE was performed with the buffer system of Laemmli (42). The gels were stained with zinc acetate for bilin chromophores (43) and subsequently with Coomassie Brilliant Blue for the protein detection.

*Spectroscopy*—The chromophorylation of phycobiliproteins was analyzed by UV-visible absorption (model Lambda 25, PerkinElmer Life Sciences, Shanghai, China, or model UV-2401PC, Shimadzu, Duisburg, Germany), circular dichroism (model J-810 spectropolarimeter, JASCO, Shanghai, China), and fluorescence spectroscopy (PerkinElmer Life Sciences model LS45 or model LS-50B). Fluorescence of PCB-chromoproteins could be detected by the emission at 630 or 645 nm (19, 40), of PEB-chromoproteins at 575 nm (20), of PUB-chromoproteins at 505 nm (44, 45), and of PVB-chromoproteins at 580 nm (46).

*PCB, PEB, and Protein Concentration Determinations*—The covalently bound chromophores in phycobiliproteins were quantified by absorption spectroscopy in acidic urea (8 M, pH 1.5) (13) using the molar extinction coefficients of PCB at 662 nm ($\epsilon$ = 35,500 $M^{-1}cm^{-1}$) (47), of PEB at 550 nm ($\epsilon$ = 42,800 $M^{-1}cm^{-1}$) (48), of PUB at 495 nm ($\epsilon$ = 104,000 $M^{-1}cm^{-1}$) (49), and of PVB at 590 nm ($\epsilon$ = 38,600 $M^{-1}cm^{-1}$) (50). Protein concentrations were determined according to the Bradford protein assay (51), using bovine serum albumin as standard.

*Analyses of Covalently Bound PUB or PVB*—To determine the covalently bound PUB or PVB, the purified and dialyzed chromophorylated phycobiliproteins were digested with trypsin. The chromopeptides derived were separated and spectroscopically analyzed via HPLC with a diode array absorption detector (Tidas, J&M Analytik AG, Aalen, Germany) (20). The purified chromopeptides were measured by mass spectrometry in positive ion mode using a Q-Tof Premier mass spectrometer (Waters Micromass Technologies, Manchester, UK) with a nano-electrospray mass ionization source (20).

## RESULTS AND DISCUSSION

*A Novel Trichromatic Phycocyanin in Synechococcus sp. WH8102*—The concomitant presence of *rpcG*, *cpcS*, and *rpcT* homologs in the genome of *Synechococcus* sp. WH8102 (as well as in three other sequenced, oceanic strains, see above), led us to postulate that this strain may possess a novel phycocyanin binding a PUB at α-84, a PCB at β-82, and possibly a PEB at β-153. To verify this hypothesis, we isolated PBSs from *Synechococcus* sp. WH8102, separated the different phycobiliproteins by non-denaturing IEF, and then analyzed them by *in situ* absorption and fluorescence spectroscopy. As expected, the phycocyanin fraction exhibited three absorption maxima at 490, 534, and 615 nm, indicating the presence of three different chromophores, PUB, PEB, and PCB, respectively (Fig. 2, *A* and *B*). Excitation into all three bands resulted in an emission of the lowest energy chromophore, PCB (supplemental Fig. S1), indicating the efficient coupling of the three chromophores. We propose to call this novel phycocyanin R-PC V following the nomenclature of Sidler (5). Fractionation into its constitutive subunits by IEF under denaturing conditions resulted in an R-PC II-like β-subunit containing one PCB and one PEB chromophore, and an α-subunit containing a PUB chromophore ($\lambda_{max}$ = 492 nm, Fig. 2, *C* and *D*); this result is consistent with the prediction made from comparative genomics. Mass spectrometry analyses of these fractions unambiguously identified the expected phycocyanin α- and β-subunits from WH8102 (supplemental Table S3 and supplemental Fig. S2). Although not direct evidence, these results strongly support our previous hypothesis that the *rpcT* gene may encode a phycocyanin β-153 PEB lyase (7). This is further confirmed by the fact that CpcT (encoded by the *cpcT* gene, a paralog of *rpcT*) can add either PCB or PEB to cysteine-β153 when expressed in *E. coli* (Table 1), although it binds only PCB in the cyanobacterium from which the gene is derived.

*A Biochemical Pathway to Phycourobilin*—The finding of PUB on *Synechococcus* sp. WH8102 phycocyanin α-subunit and its correlation with the *rpcG* gene strongly suggested that the latter encoded an enzyme catalyzing the concomitant biosynthesis of the PUB chromophore from a PEB precursor and its attachment to cysteine α-84 of R-PC V. Direct proof for this was obtained by using an adapted (20) multiplasmid expression system in *E. coli* (52). Unlike *in vitro* chromophorylations of

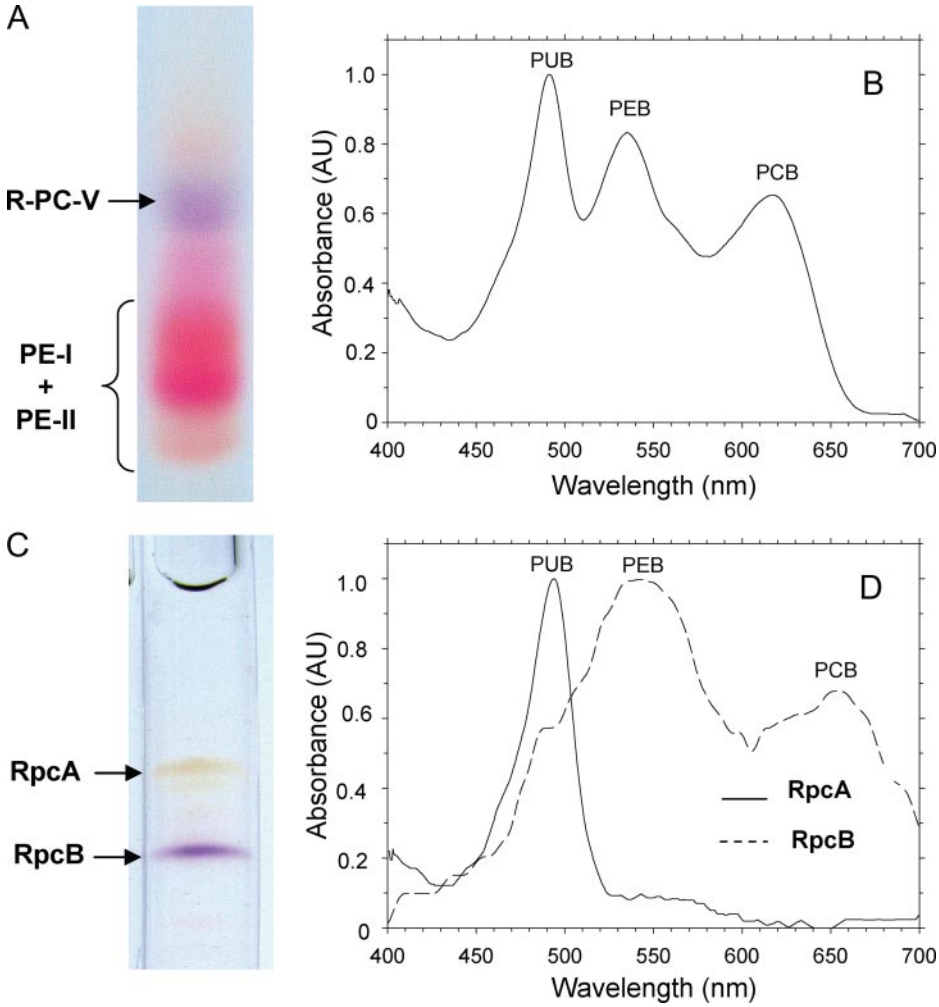


FIGURE 2. **Isolation and properties of R-PC V from *Synechococcus* sp. WH8102.** *A*, components of phycobilisomes, separated by non-denaturing isoelectric focusing on polyacrylamide gels; the more acidic allophycocyanins were lost under these conditions. *a.u.*, absorbance units. *B*, absorption spectrum of R-PC V. *C*, subunits of R-PC V separated by denaturing isoelectric focusing. *D*, absorption spectra of isolated subunits.

biliproteins, the *E. coli* system is largely devoid of unspecific side reactions and avoids solubility problems (18). The *rpcG* gene was expressed together with the gene for the apoprotein, RpcA, and three genes coding for enzymes that generate the PEB chromophore from heme, *viz.* heme oxygenase (*ho1*) and two bilin reductases (*pebA* and *pebB*) (26). The balanced gene expression of the introduced genes can be controlled by growth at a relatively low temperature (16–20 °C).

This system indeed generated the desired chromoprotein, termed PUB-RpcA$_{8102}$. No chromophorylated RpcA was formed if the *rpcG*-containing plasmid was omitted. Purified native PUB-RpcA$_{8102}$ exhibited the characteristic absorption, fluorescence, and circular dichroism spectra of PUB-containing phycobiliproteins (Fig. 3, Table 1, and supplemental Fig. S3). Formation of the PUB chromophore was verified by the spectra of both the denatured protein (supplemental Fig. S3) and the chromopeptides obtained by tryptic digestion (supplemental Fig. S4). Covalent attachment of the PUB chromophore was confirmed by the $Zn^{2+}$-induced green fluorescence on denaturing gels (Fig. 4) and correct binding to cysteine 84 by tryptic digestion and HPLC/mass spectrometry (supplemental Table S4

**TABLE 1**

**Quantitative absorption and fluorescence data of reconstituted biliproteins**

Although every reaction was tested with reductases generating PEB or, in most cases PCB, and with a combination of each lyase and apoprotein that are listed in supplemental Table S2, and under "Experimental Procedures," only those reactions are listed that resulted in successful chromophorylation. Spectra were obtained in potassium phosphate buffer (20 mM, pH 7.2) containing NaCl (0.5 M), and data were averaged from 2–3 independent experiments. $Q_{Vis/uv}$ denotes the absorbance ratio of the visible and near-UV bands.

| Biliprotein | Lyase | Absorption | | Fluorescence ($\lambda_{max}$) |
|---|---|---|---|---|
| | | $\lambda_{max}$ ($Q_{Vis/uv}$) | $\epsilon_{Vis}$ | |
| | | *nm* | $M^{-1} \cdot cm^{-1}$ | *nm* |
| PUB-PecA[a] | RpcG[b] or PecE/F[a] | 496/374 (8.6) | 1.54 (±0.08) × $10^5$ | 505 |
| PUB-RpcA[b] | RpcG[b] | 492/376 (6.7) | 1.58 (±0.01) × $10^5$ | 502 |
| PUB-RpcA[c] | RpcG[c] | 493/375 (11.9) | 1.27 (±0.04) × $10^5$ | 503 |
| PVB-PecA[a,d] | RpcG[b] or PecE/F[a] | 565/331 (2.6) | 0.98 (±0.01) × $10^5$ | 582 |
| PVB-RpcA[b,d] | RpcG[b] | 557/329 (3.2) | 0.95 (±0.03) × $10^5$ | 577 |
| PEB-CpcA[a] | CpcE/F[a] | 555/381 (3.6) | 1.39 (±0.03) × $10^5$ | 568 |
| PEB-PecA[a] | CpcE/F[a] | 555/379 (6.4) | 1.30 (±0.04) × $10^5$ | 568 |
| PEB-CpcB(C84S)[e] | CpcT[a] ± (RpcG[b] or PecE/F[a]) | 533/384 (5.5) | 0.92 (±0.04) × $10^5$ | 552 |
| PEB-CpcB(C155I)[e] | CpcS[a] ± (RpcG[b] or PecE/F[a]) | 555/371 (6.9) | 1.33 (±0.03) × $10^5$ | 568 |
| PEB-PecB(C84A)[e] | CpcT[a] ± (RpcG[b] or PecE/F[a]) | 537/394 (3.0) | 0.94 (±0.02) × $10^5$ | 554 |
| PEB-PecB(C155I)[e] | CpcS[a] ± (RpcG[b] or PecE/F[a]) | 546/381 (7.6) | 0.98 (±0.07) × $10^5$ | 562 |

[a] Expressed genes were from *Nostoc* sp. PCC7120.
[b] Expressed genes were from *Synechococcus* sp. WH8102.
[c] Expressed genes were from *Synechococcus* sp. RS9916.
[d] Amplitudes (ΔΔA, see Ref. 46) of the reversible photochemistry of PVB-PecA and PVB-RpcA formed in the presence of RpcA were 103% and 22%, respectively.
[e] Expressed genes were from *Fischerella* sp. PCC7603.

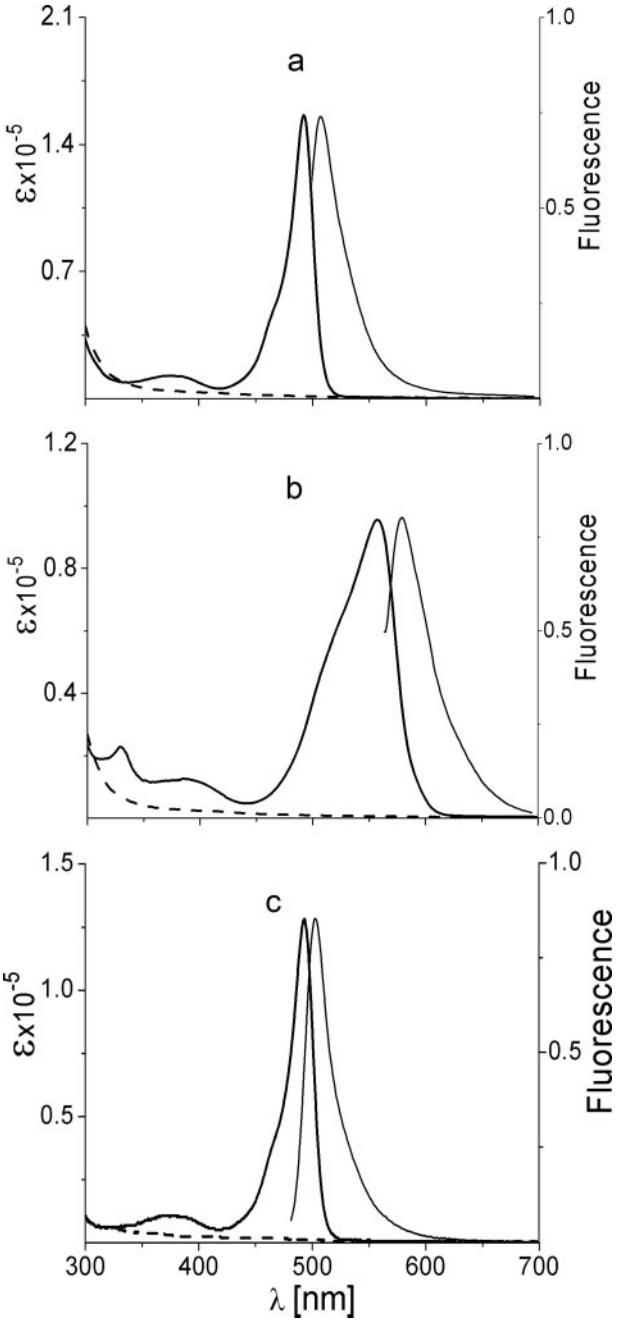


FIGURE 3. **Absorption (*heavy lines*) and fluorescence spectra (*thin lines*) of $Ni^{2+}$ affinity-purified PUB-$RpcA_{8102}$ (*a*), PVB-$RpcA_{8102}$ (*b*), and PUB-$RpcA_{9916}$ (*c*) in potassium phosphate buffer (20 mM, pH 7.2) containing NaCl (0.5 M) and imidazole (0.5 M (*a* and *b*) or 0.2 M (*c*)).** Chromoproteins were reconstituted in *E. coli* under catalysis of RpcG. Controls (*dashed lines*) from samples produced in the absence of RpcG were nearly free of chromoproteins. Fluorescence from PUB was excited at 470 nm, and that from PVB was excited at 540 nm. The subsequent HPLC of chromopeptides obtained by tryptic digestion of PUB-RpcA is shown in supplemental Fig. S4.

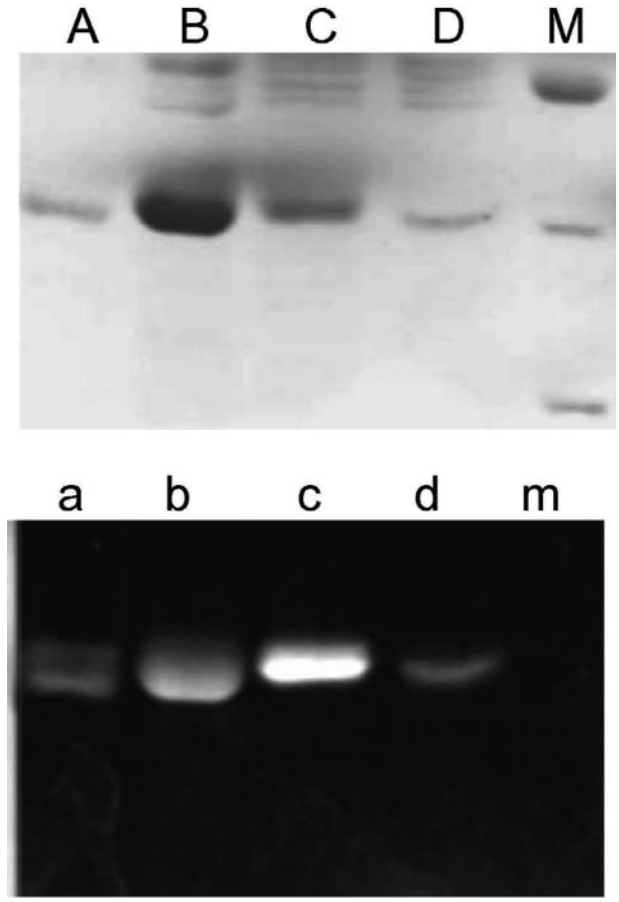


FIGURE 4. **SDS-polyacrylamide gel electrophoresis of His-tagged chromoproteins.** PUB-PecA (*lanes A* and *a*), PUB-RpcA (*lanes B* and *b*), PVB-PecA (*lanes C* and *c*), and PVB-RpcA (*lanes D* and *d*), detected by staining with Coomassie Blue (*top*) and $Zn^{2+}$-induced fluorescence (43) (*bottom*). *Lane M*, protein markers (from *top* to *bottom*: 24, 20, and 14 kDa). The samples were obtained via chromophorylation in *E. coli* and subsequent purification with $Ni^{2+}$-affinity chromatography (see "Experimental Procedures").

and supplemental Figs. S4 and S5). Additional support for the function of RpcG was the formation of the homologous chromoprotein, PUB-$RpcA_{9916}$, when the respective genes (*rpcA*, *rpcG*, *pebA*, *pebB*, and *ho1*), from *Synechococcus* sp. RS9916, were introduced into the heterologous *E. coli* system (Fig. 3*c*).

*The Lyase-Isomerase RpcG Can Use Different Substrates*—To further test the isomerase capacities of RpcG, we replaced *pebA* and *pebB* genes in the *E. coli* system by *pcyA*, encoding the PCB:ferredoxin oxidoreductase, an enzyme that catalyzes the two-step reduction of biliverdin to produce PCB (53). When the phycoerythrocyanin $\alpha$-subunit PecA from *Nostoc* sp. PCC 7120 was provided as the acceptor protein, the PCB chromophore was not only attached to at the correct $\alpha$-84 site but also isomerized at the same time to the PVB chromophore. The product, PVB-PecA, had the characteristic spectral properties of PVB-phycobiliproteins; it showed, in particular, the unique reversible orange-green photochemistry (46) (Table 1 and supplemental Fig. S6*b*). The difference spectrum of this photoreaction is qualitatively and quantitatively identical to that of $\alpha$-PEC from *Nostoc* sp. PCC 7120 generated by using the isomerizing lyase PecE/F and the apoprotein PecA from the same organism (54). Moreover, RpcG could also attach PCB to RpcA with concomitant isomerization to PVB, when the system was co-transformed with the *rpcA* gene from *Synechococcus* sp. WH8102. The product (PVB-RpcA) again showed the characteristic spectra and photochemistry (Table 1, Fig. 3*b*, and supplemental Fig. S6*a*). However, the amplitude in this case was only ~20% of that of the authentic $\alpha$-PEC, probably reflecting the hybrid situation when using the apoprotein from *Synechococcus* sp. WH8102 that naturally carries a PUB chromophore. There was again no such product formed in the absence of the

*rpcG* gene. It is clear from these results that RpcG, in the heterologous system, can use both PEB and PCB as substrates and attach them, with concomitant isomerization to PUB and PVB, respectively, to cysteine 84 of phyco(erythro)cyanin α-subunits (Fig. 1). It combines, therefore, the functions of the two subunits of the isomerizing-lyase, PecE/F, which catalyze the double bond shift of (Δ4→Δ2) in both PEB and PCB. As mentioned previously, RpcG contains the NHCQGN sequence that has been identified as a critical motif for isomerizing lyases that accept PCB (35), suggesting that the isomerase function of this motif extends to PEB as substrate. It should be noted, however, that despite using PCB in the *E. coli* system, RpcG does not use PCB in natural conditions because no PVB has been observed in *Synechococcus* sp. WH8102, nor has it ever been reported from any other *rpcG*-containing strains. This restricted specificity *in vivo* may indicate that additional factors control the lyase specificities, such as timing or kinetics.

*Re-examining the Properties of the Isomerizing Lyase PecE/F*—The acceptance, in *E. coli*, of both PCB and PEB as substrates by RpcG prompted us to reinvestigate the substrate and the acceptor-protein specificities of the heterodimeric isomerizing lyase PecE/F. The respective genes, *pecE/F*, from *Nostoc* sp. PCC 7120 were again expressed in the *E. coli* system (20, 52). Providing PEB as chromophore and PecA as acceptor protein, PUB-PecA was obtained as the major product. In the absence of PecE/F, only small amounts of a chromoprotein were formed, and these contained the non-isomerized PEB chromophore, as judged from its absorption properties. Absorption, fluorescence, and circular dichroism spectra of PUB-PecA formed under the action of PecE/F were similar to those of PUB-RpcA (Fig. 5 and Table 1); the chromophore was covalently attached according to denaturing gel electrophoresis and $Zn^{2+}$ staining (Fig. 4), and cysteine 84 was identified as the binding site by mass spectroscopy of tryptic peptides (supplemental Figs. S7 and S8 and supplemental Table S4). No PUB chromoproteins were formed when phycocyanin β-subunits or PE α-subunits were offered as acceptor proteins together with PecE/F alone or in combination with other lyases (Table 1). If any chromoproteins were formed in these cases, they contained non-isomerized PEB chromophore. The isomerization reaction therefore requires cooperation of two matching partners, an isomerizing lyase and an appropriate acceptor protein, namely, PecA or RpcA.

We have demonstrated here, for the first time, a biosynthetic pathway to the PUB chromophore in phycobiliproteins. It is analogous to the formation of PVB; in both cases, the same isomerization occurs at ring A during the attachment reaction, and the lyases, chromophores, and accepting proteins can be interchanged as long as an α-subunit of a phycocyanin or phycoerythrocyanin is provided (Fig. 1). The emerging modular properties of the E/F-type lyases are reminiscent of those of the bilin reductases. In the biosynthesis of phycobiliproteins, these reductases act prior to chromophore attachment reaction and may be involved in metabolic channeling of these chromophores, which, in their free form, are chemically very labile (26).

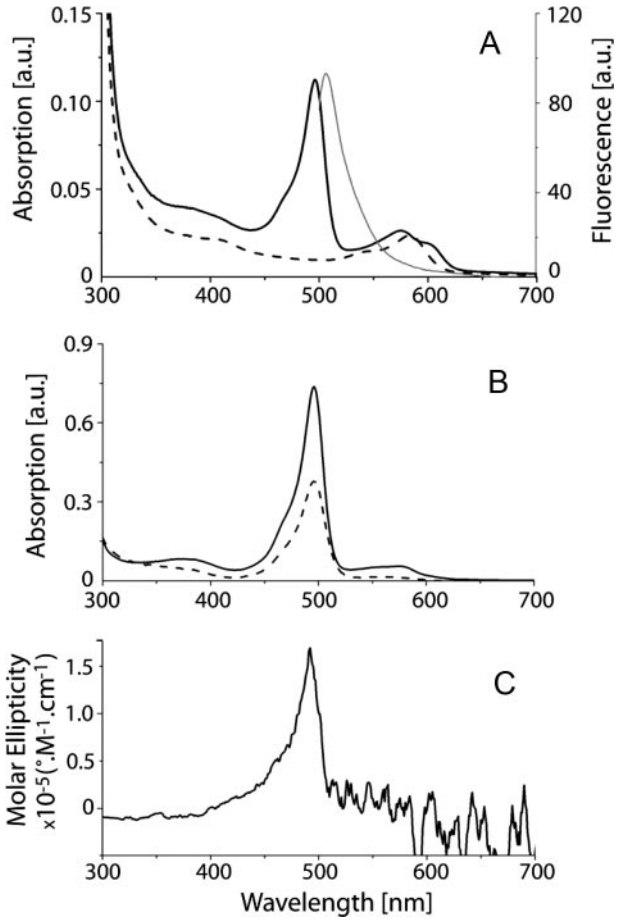


FIGURE 5. **Absorption, fluorescence and CD spectra of PUB-PecA.** Samples were reconstituted in *E. coli* and purified by $Ni^{2+}$ affinity chromatography and FPLC (see "Experimental Procedures"). *A*, absorption (*heavy lines*) and fluorescence (*thin line*) of PUB-PecA in potassium phosphate buffer (20 mM, pH 7.2) containing imidazole (0.5 M) and NaCl (0.5 M). In the presence of PecE/F (*solid line*), the major chromophorylation product was PUB-PecA. It was accompanied by unidentified chromoprotein(s) that are formed by a side reaction in the absence of PecE/F (control, *dashed line*). For clarity, only fluorescence from PUB (excitation 470 nm) was displayed. The subsequent HPLC after tryptic digestion is shown in supplemental Fig. S8; *a.u.*, absorbance units. *B*, absorption (*top*) and CD-spectra (*bottom*). *Solid lines* are from native sample in potassium phosphate buffer (20 mM, pH 7.2) containing NaCl (0.5 M), and the *dashed line* is from the denatured sample in acidic urea (8 M, pH 1.9).

*Physiological, Evolutionary, and Biotechnological Implications of R-PC V*—PUB is the major chromophore in the PBSs of marine *Synechococcus* adapted to oceanic waters. It should be noted, however, that the R-PC V studied here binds only a minor fraction of PUB in these strains, whereas the majority is contained in phycoerythrins (Fig. 2). Further work is therefore needed to show whether there are homologous enzymes involved in the biosynthesis of these phycoerythrin-bound PUB molecules. Having obtained the first such enzyme and others known for the three other chromophores (18), we believe that it is very likely that progress in identifying the lyases responsible for these binding sites will be helped. The identification of RpcG as a phycourobilin:phycocyanin-α84-cystein-lyase (isomerizing) then seems to be a critical milestone in understanding PBS assembly. The biosynthetic scheme derived from these results is summarized in Fig. 1. The fused lyase may be better suited for crystallizations, which failed with the heterodimeric homologues.

From an evolutionary viewpoint, the trichromatic R-PC V is remarkable because it is the only biliprotein (or even chromoprotein in general) so far known to harvest the complete range of wavelengths between the two peaks of chlorophyll *a* at ~440 and ~675 nm. Presently, one can only speculate on the possible functions of this unusual pigmentation. It probably contributes little additional light absorption because the majority of PUB is contained in PE I and PE II (Fig. 2) (37). Because PCs are located at the base of PBS rods, they are critical in transferring excitation energy to the PBS core; a function in energy transfer is therefore more likely. In PUB-rich PBSs, the presence of R-PC V with both PUB and PEB chromophores may improve spectral overlap with both the high energy PUB and the intermediate energy PEB in the distal parts of the rods, thereby optimizing energy equilibration and the further transfer to the low energy PCB chromophores of the allophycocyanins in the PBS core and, ultimately, to the RCs. An *rpcG*-like gene is also present in the cyanobacterium, *Crocosphaera watsonii* (strain WH8501), which is particularly rich in PUB but phylogenetically very distant from marine *Synechococcus* (25). A function in energy transfer would rationalize the presence of a PUB-carrying $\alpha$-subunit in its type IV R-PC (5, 27). A transfer-optimizing function at the rod/core interface would also rationalize the unique variety in pigmentation of PC $\alpha$-subunits; Cys-$\alpha$84 is the only binding site that carries, in different organisms, all four different chromophores of cyanobacterial phycobiliproteins. Identification of the trichromatic R-PC V and the first PUB-lyase, RpcG, may also assist engineering fluorescence labels with Stokes shifts as large as 165 nm (supplemental Fig. S1), thereby extending phycobiliprotein applications in medicine and life sciences.

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